

HIV-Nef and ADAM17-containing plasma extracellular vesicles induce and correlate with immune pathogenesis in chronic HIV infection

Supplementary Information:

Human cytokine/chemokine/soluble factor (CCF) array

Purified EV from sucrose gradient fractions were applied to the RayBio Human Cytokine Array C-S (Hölzel Diagnostika, AAH-CYT-1000-2) according to the manufacturer's instructions. Cytokines were analyzed and identified based on the following table:

Human Cytokine Antibody Array C6

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
2	POS	POS	NEG	NEG	BLANK	ANG	BDNF	BLC	BMP 4	BMP 6	CCL23	CNTF	EGF	Eotaxin 1
3	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
4	Eotaxin 2	Eotaxin 3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM CSF	I-309	IFN gamma	IGFBP 1	IGFBP 2	IGFBP 4
5	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
9	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS
10	PDGF BB	RANTES	SCF	SDF-1 alpha	TARC	TGF beta 1	TGF beta 3	TNF alpha	TNF beta	BLANK	BLANK	BLANK	BLANK	POS

Human Cytokine Antibody Array C7

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	BLANK	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	b-NGF	BTC	CCL28
2	POS	POS	NEG	NEG	BLANK	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	b-NGF	BTC	CCL28
3	CTACK	Dtk	EGFR	ENA-78	Fas	FGF-4	FGF-9	G-CSF	GITR Ligand	GITR	GRO	GRO alpha	HCC-4	HGF
4	CTACK	Dtk	EGFR	ENA-78	Fas	FGF-4	FGF-9	G-CSF	GITR Ligand	GITR	GRO	GRO alpha	HCC-4	HGF
5	ICAM-1	ICAM-3	IGFBP 3	IGFBP 6	IGF-1 sR	IL-1 R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
6	ICAM-1	ICAM-3	IGFBP 3	IGFBP 6	IGF-1 sR	IL-1 R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
7	I-TAC	XCL1	MIF	MIP-1 alpha	MIP-1 beta	MIP-3 beta	MSP alpha	NT-4	OPG	OSM	PLGF	sgp130	sTNFRII	sTNFRI
8	I-TAC	XCL1	MIF	MIP-1 alpha	MIP-1 beta	MIP-3 beta	MSP alpha	NT-4	OPG	OSM	PLGF	sgp130	sTNFRII	sTNFRI
9	TECK	TIMP-1	TIMP-2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	BLANK	BLANK	BLANK	BLANK	POS
10	TECK	TIMP-1	TIMP-2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	BLANK	BLANK	BLANK	BLANK	POS

Supplementary Figures S1-S6

Figure S1:

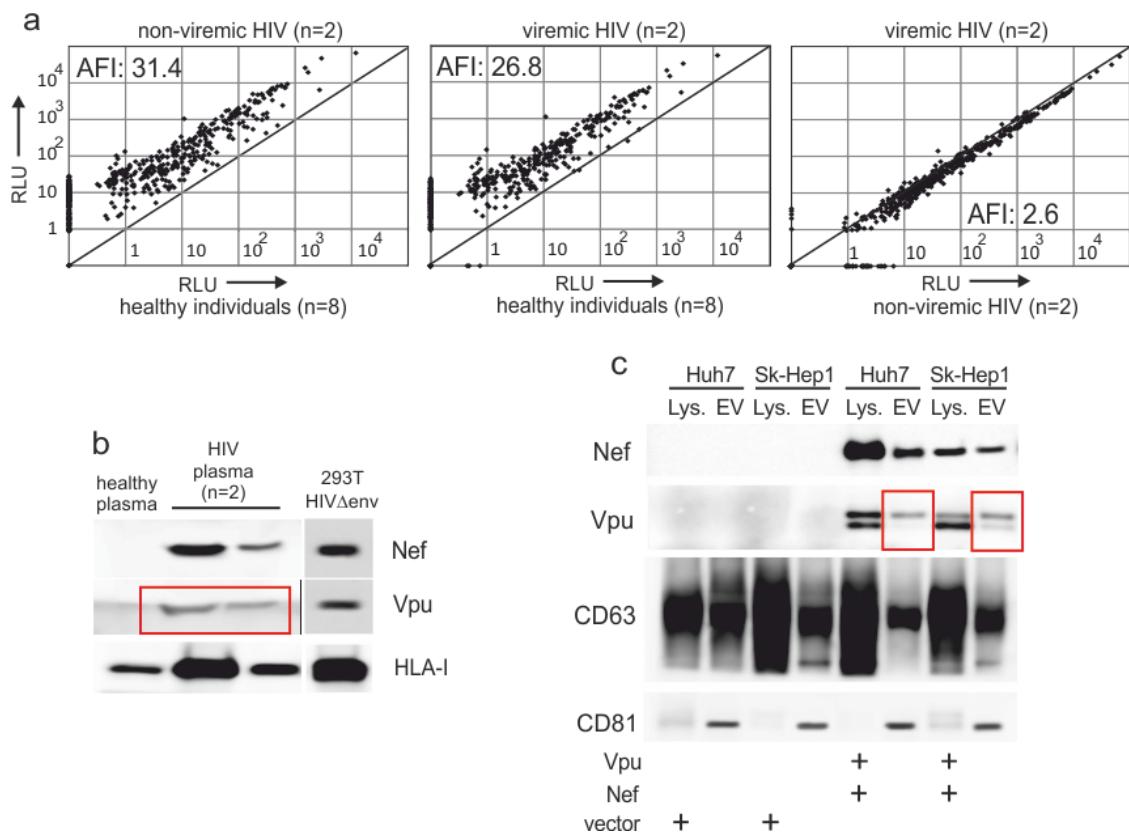
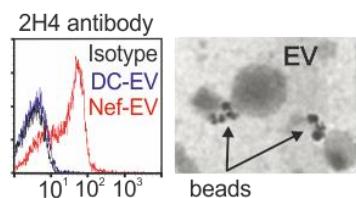


Figure S1: Elevated pEV-derived miRNAs in viremic and non-viremic HIV patients and presence of Vpu in HIV pEV. (A) Pairwise comparison of pEV miRNA levels derived from 2 non-viremic and 2 viremic HIV patients and 8 healthy controls, performed as in Figure 1. Each dot depicts the relative concentration of one miRNA. In this analysis the mean of two miRNA data sets ($n=2$; viremic or non-viremic) was compared with the mean of 8 data sets from healthy individuals. AFI: Average fold-increase of all miRNAs over reference. (B) pEV from two non-viremic plasma samples (3ml) were purified and analyzed by Western blot for the indicated proteins. For antibody reactivity control 293T cells were transfected with a HIV Δ env viral construct and blotted accordingly. (C) Huh7 and Sk-Hep-1 liver cells were transiently transfected with Nef and Vpu expression plasmids as indicated. After 48h, EV were purified from supernatants and blotted for Nef and Vpu along with conventional EV markers (CD63, CD81). For comparison cell lysates were blotted side by side.

Figure S2.

a



b

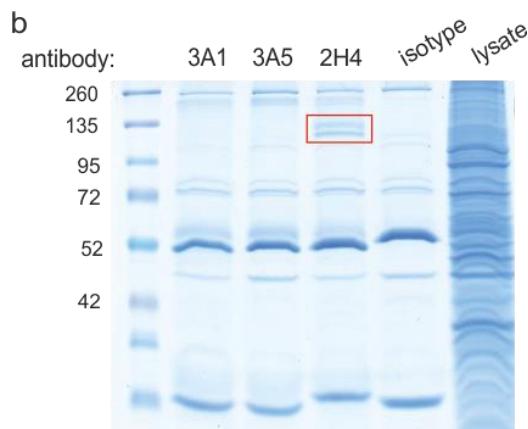


Figure S2: Detection of 2H4 for Nef pEV immunisolation and MALDI TOF Analysis. (a) Screening and bead-coupling of an antibody specifically recognizing HIV pEV. Dendritic cell (DC)-derived EV and Nef-EV (purified from 293T cell culture supernatants after transfection of Nef) were coupled to polystyrene beads and analyzed by FACS using antibody hybridomas (here 2H4). The selected 2H4 antibody was coupled to magnetic micro-beads by Miltenyi Biotec. For control, electron micrographs were taken as previously described (Lee et al., 2013). **(b)** Several monoclonal antibodies generated to recognize EV antigens, including 2H4, were used to immunoprecipitate their cognate antigen from Huh7 lysates. The result was visualized by coomassie blue staining. The indicated band (red box) was subsequently used for mass spectrometer resolution analysis (MALDI-TOF).

Figure S3:

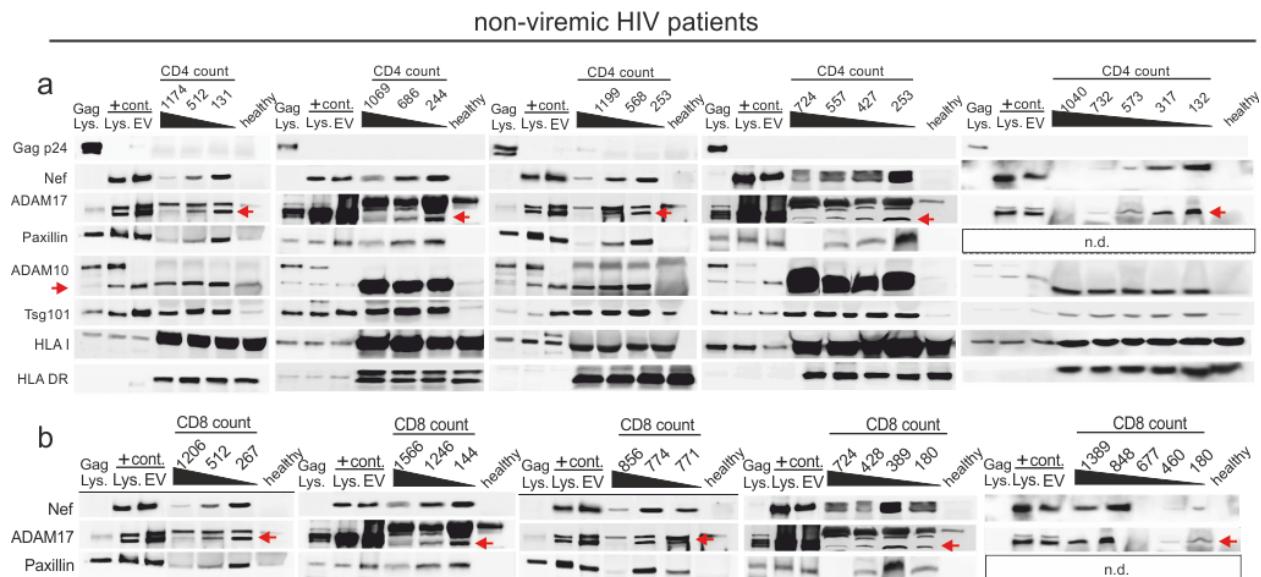


Figure S3: pEV-derived Nef and ADAM17 correlate with low CD4 counts (non-viremic patients). Correlation of pEV protein levels of Nef and cellular markers with CD4 (**A**) and CD8 count (**B**). Plasma-EV were purified from 5 ml plasma of non-viremic HIV patients by differential centrifugation. The pEV equivalent of 3 ml plasma was analyzed by Western blot. Lysates (Lys.) of 293T cells, transfected with p24 Gag (Gag Lys.) or Nef/Nef-cofactors (+cont.) served as controls, as previously described in (2), as well as EV purified from these transfected cells (EV). Red arrows depict the activated form of ADAM17.

Note: the blots in (**B**) are identical to those in (**A**), but were rearranged with respect to decreasing CD8 counts. Also note that the first panel in (**A**) is identical to the left panel in Figure 2A.

Figure S4:

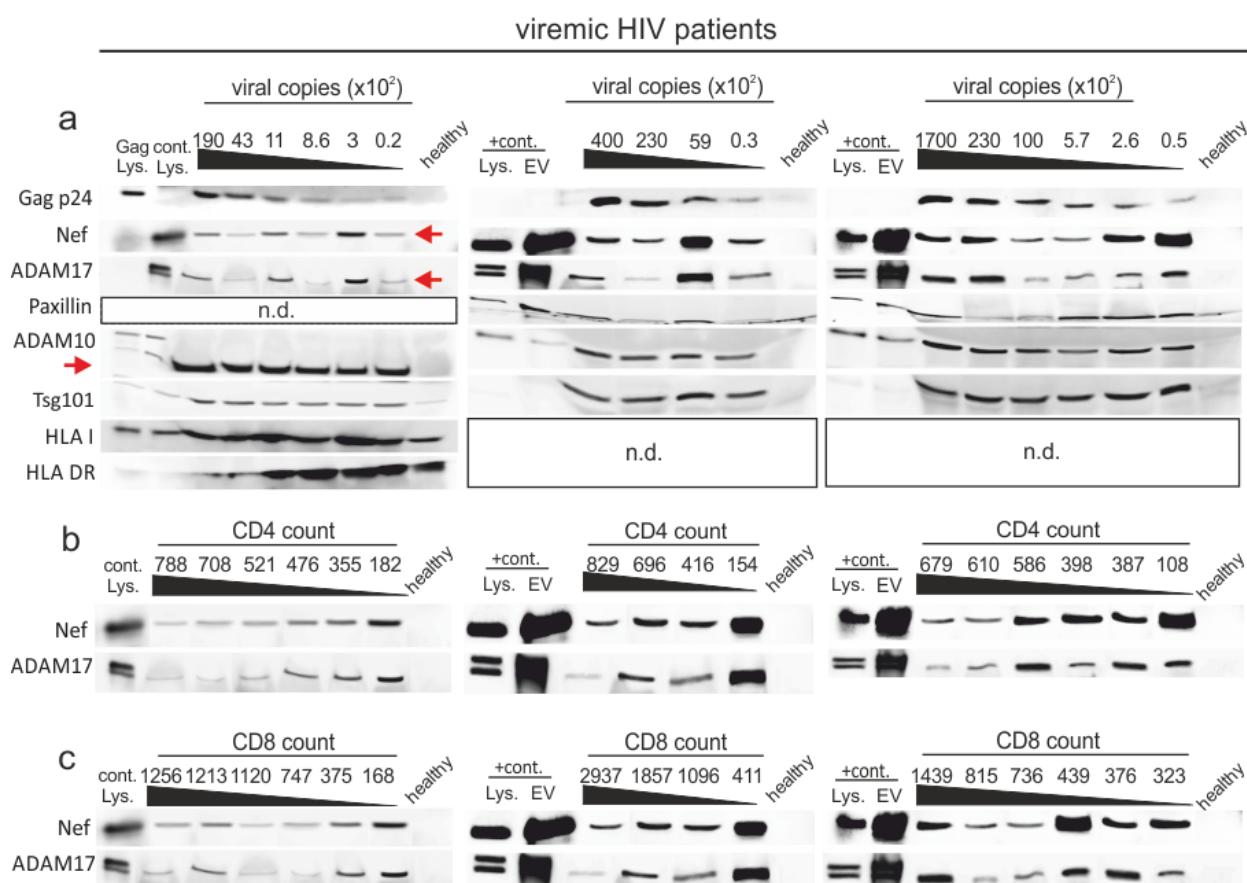


Figure S4: Plasma EV-derived Nef and ADAM17 protein levels correlate with low CD4 and CD8 counts (viremic patients). Correlation of pEV protein levels of Nef and cellular markers with viral copy numbers (**A**), CD4 count (**B**) and CD8 count (**C**). pEV were purified by differential centrifugation from 5 ml plasma from viremic HIV patients. The pEV equivalent of 3 ml plasma was analyzed by Western blot. Lysates (Lys.) of 293T cells, transfected with p24 Gag (Gag Lys.)

or Nef/Nef-cofactors (+cont.), served as controls, as well as EV purified from these transfected cells (EV).

Note: the blots in (B) and (C) are identical to those in (A), but were rearranged with respect to decreasing CD4 and CD8 counts. Also, the first panel in (A) is identical to the right panel in Figure 2A.

Figure S5:

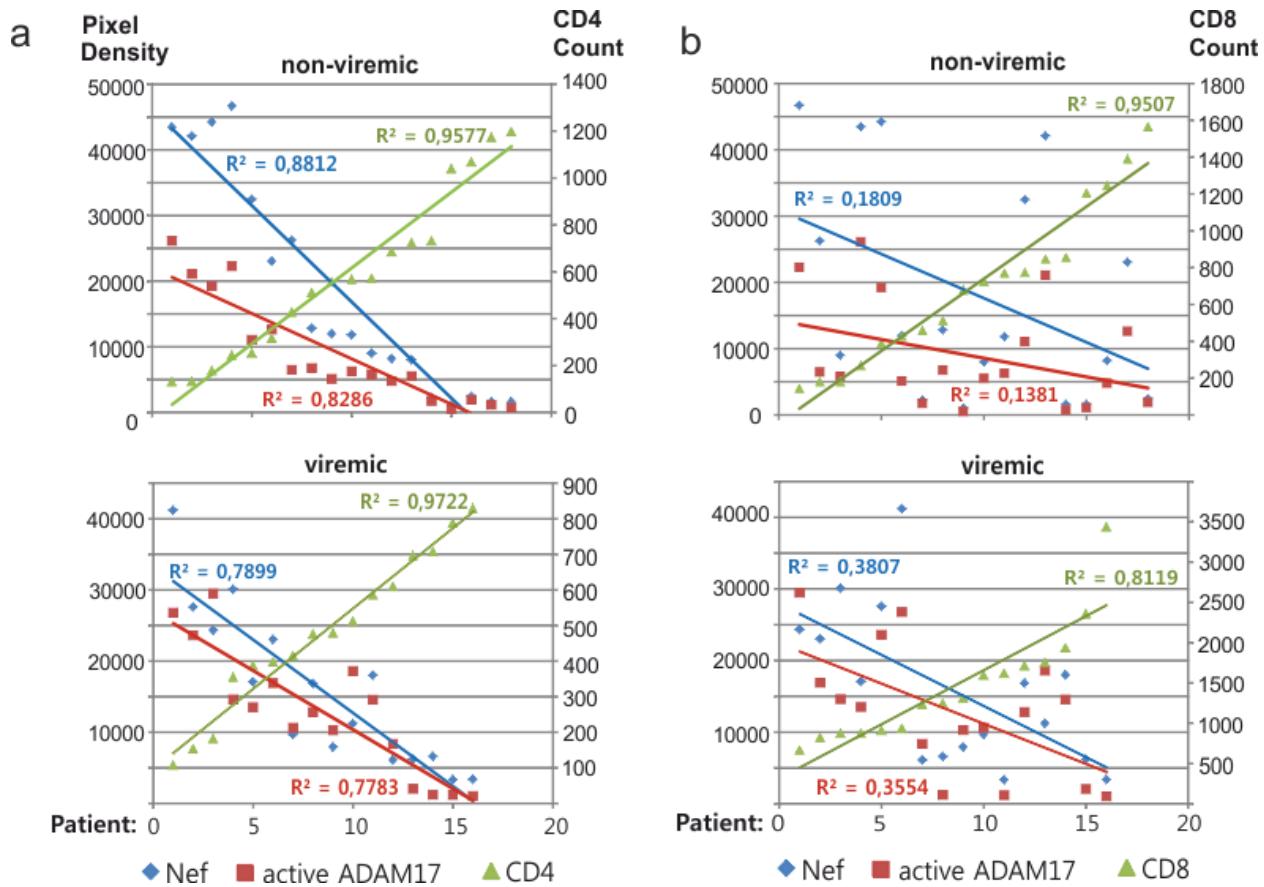


Figure S5: pEV-derived Nef and ADAM17 protein levels correlate with low CD4 (viremic and non-viremic patients) and CD8 counts (viremic patients). Correlation of relative protein levels of Nef and active ADAM17 (determined as relative pixel density by ImageJ in Western blots shown in fig. S2 and S3) and CD4- (A) and CD8 counts (B) in viremic and non-viremic patients as indicated.

Figure S6:

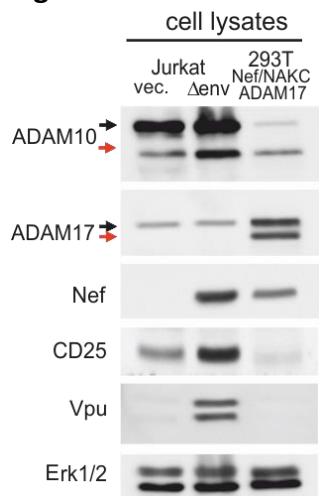


Figure S6: Nef/Vpu expression control in Jurkat cells. Transient transfection of HIV- Δ env or empty vector into Jurkat T cells. After 72h cells were harvested and EV were purified from cell culture supernatants. EV and cell lysates were blotted for the indicated markers. Nef/co-factor-transfected 293T cells served as positive controls.

Table S1:

Plasma:

Patient code	Viral copies/ml	CD4 counts/ μ l	CD8 counts/ μ l	Figure	Patient code	Viral copies/ml	CD4 counts/ μ l	CD8 counts/ μ l	Figure	
1	200GJ	1300	1014	893	Fig. 1 a	61	566V-2	10000	679	815
2	871MS	1400	364	877	Fig. 1 a	62	1209TT	23000	586	1439
3	365MMS	9100	194	971	Fig. 1 a	63	1285BH	170000	387	376
4	0647NT	1300	357	821	Fig. 1 a	64	099KI-1	<20	244	144
5	0081BA-1	<20	479	494	Fig. 1 a	65	947AS-1	<20	253	774
6	0924HR-1	<20	391	647	Fig. 1 a	66	0008AS-1	<20	178	389
7	0934LT	<20	593	505	Fig. 1 a	67	0678C	<20	724	724
8	1477TA	<20	577	510	Fig. 1 a	68	0963CJ	<20	1040	677
9	3810W	<20	428	571	Fig. 1 b / Sup. Fig. 1 a	69	0666BB-1	<20	686	1246
10	198HU	<20	594	537	Fig. 1 b / Sup. Fig. 1 a	70	0006AC	<20	1069	1566
11	537TM	5600	757	639	Fig. 1 b / Sup. Fig. 1 a	71	451SH-2	<20	568	771
12	451SH-1	15000	412	841	Fig. 1 b / Sup. Fig. 1 a	72	293KP-1	<20	1199	856
13	303LT-1	<20	441	520	Fig. 1 c	73	099KI-2	<20	427	180
14	303LT-2	<20	444	590	Fig. 1 c	74	034BP	<20	596	373
15	228H	<20	460	532	Fig. 1 c	75	1345SM	<20	132	848
16	127FP-1	<20	352	626	Fig. 1 c	76	1083WH-1	<20	317	1389
17	1372SH	3500	181	1135	Fig. 1 c	77	099KI-3	<20	573	180
18	903HE	80000	353	418	Fig. 1 c	78	0485G	<20	732	460
19	995PG	1700	593	491	Fig. 1 c	79	145FP	20	521	1256
20	140FM	6700	463	827	Fig. 1 c	80	248A	300	182	168
21	918SB	<20	351	341	Fig. 1 d / Fig. 2 a	81	369NB-2	860	708	747
22	189HT	<20	389	576	Fig. 1 d / Fig. 2 a	82	517SD	1100	355	375
23	0557VP	<20	341	902	Fig. 1 d / Fig. 2 a	83	1322WT	4300	788	1120
24	368NB	<20	338	454	Fig. 1 d / Fig. 2 a	84	0643GA-1	19000	476	1213
25	048BW-2	<20	468	1427	Fig. 1 e	85	1190P	30	416	1096
26	355MJ-2	<20	533	722	Fig. 1 e	86	1303HT	2900	154	411
27	153GT	<20	1202	560	Fig. 1 e	87	010AJ	23000	829	2937
28	0924HR-2	<20	332	1183	Fig. 1 e	88	0634GA-2	40000	696	1857
29	372NE	<20	902	513	Fig. 1 e	89	1083WH-2	<20	80	944
30	11AB	<20	521	436	Fig. 1 e	90	947AS-2	<20	210	930
31	562VH	<20	710	1725	Fig. 1 e	91	331ME-1	<20	182	820
32	104DT	<20	588	532	Fig. 1 e	92	042BR	<20	561	201
33	1048ES	<20	151	590	Fig. 1 e	93	0668B-2	<20	672	1158
34	1011PA-1	59000	464	1938	Fig. 2 a	94	069BG-1	<20	476	494
35	566V-1	23000	444	713	Fig. 2 a	95	069BG-2	<20	491	372
36	1078LR	33887	560	1304	Fig. 2 a	96	0488BV-1	<20	542	2166
37	1014FG-1	3100	873	1714	Fig. 2 a	97	41GRH	<20	714	460
38	365MV	9100	194	971	Fig. 2 b	98	0478BM-1	<20	766	198
39	1410WW	180	25	1219	Fig. 2 b	99	0478M-2	<20	919	301
40	0008FS	1400	285	818	Fig. 2 b	100	050BR-2	<20	842	689
41	1433KH	2100	189	859	Fig. 2 b	101	1014FG-2	<20	1318	1791
42	0942NK	<20	925	523	Fig. 2 b	102	366NN-2	<20	158	1106
43	370NA	<20	1097	439	Fig. 2 b	103	985EK	80	163	1557
44	050BR-1	<20	1056	816	Fig. 2 b	104	1001WK	100	267	388
45	258K-1	<20	953	782	Fig. 2 b	105	452SA	420	508	1133
46	1245MU	<20	197	877	Fig. 3 b	106	480SG	<20	794	498
47	916GS-1	<20	352	652	Fig. 3 b and d	107	984ET	<20	946	1081
48	345MM	<20	786	679	Fig. 3 b and d	108	162GS	1900	481	1002
49	846LJ	<20	359	359	Fig. 3 c	109	113SEF-1	990	166	332
50	04895M	<20	345	719	Fig. 3 c	110	113SEF-2	130	203	473
51	916GS-2	<20	349	338	Fig. 3 c	111	1538BVW	2200	244	853
52	1243AJ	<20	284	703	Fig. 3 c	112	1215RN	<20	273	758
53	243JF	<20	225	197	Fig. 4 b / Fig. 6 c	113	293KP-2	<20	1415	892
54	046BJ	<20	166	284	Fig. 4 b / Fig. 6 c					
55	1061KW-1	<20	131	267	Fig. 6 a and b / Sup. Fig. 3 a and b					
56	156GH	<20	512	512	Fig. 6 a and b / Sup. Fig. 3 a and b					
57	0029BT	<20	1174	1206	Fig. 6 a and b / Sup. Fig. 3 a and b					
58	1061KW-2	50	108	439	Fig. 6 a and b / Sup. Fig. 4 a, b, c					
59	296KR	260	398	323	Fig. 6 a and b / Sup. Fig. 4 a, b, c					
60	369NB-1	570	610	736	Fig. 6 a and b / Sup. Fig. 4 a, b, c					

PBMC:

Patient code	Viral copies/ml	CD4 counts/ μ l	CD8 counts/ μ l	Figure	Patient code	Viral copies/ml	CD4 counts/ μ l	CD8 counts/ μ l	Figure	
1	616ZJ	<20	512	654	Fig. 4 a	26	594WJ	<20	616	1062
2	156GH	<20	512	512	Fig. 4 a	27	918SB	<20	351	341
3	377NJ	<20	453	652	Fig. 4 a	28	189HT	<20	389	576
4	947AS-1	<20	253	774	Fig. 4 a	29	1061KW-1	<20	131	267
5	397PH	<20	587	900	Fig. 4 a	30	081BA-2	<20	464	606
6	562VH	<20	710	1725	Fig. 4 a	31	423RS	<20	656	875
7	330MB	<20	378	587	Fig. 4 a	32	305LA	<20	680	680
8	543TM	<20	484	948	Fig. 4 a	33	1011PA-2	<20	617	876
9	0002AN	<20	464	539	Fig. 4 a	34	11AB	<20	521	436
10	295KR	<20	497	1050	Fig. 4 a	35	485SH	<20	680	576
11	127FP-2	<20	334	641	Fig. 4 a	36	328MR	<20	376	1330
12	050BR-1	<20	1056	816	Fig. 4 a	37	360MR	<20	544	952
13	153GT	<20	1202	560	Fig. 4 a	38	0082BS	<20	539	856
14	932GF	<20	557	624	Fig. 4 a	39	451SH-2	<20	568	771
15	916GS-3	<20	350	738	Fig. 4 a	40	1051RM	<20	637	559
16	163GS	<20	300	461	Fig. 4 a	41	0300LR	<20	657	1027
17	4715A	<20	525	364	Fig. 4 a	42	143FU	<20	788	715
18	069BG-3	<20	462	382	Fig. 4 a	43	371N	<20	787	846
19	224HU	<20	664	349	Fig. 4 a	44	578WP	<20	942	1065
20	902KC	<20	385	1052	Fig. 4 a	45	258KA-2	<20	960	837
21	331ME-2	<20	218	949	Fig. 4 a	46	0552LLK	<20	965	768
22	1005KG	<20	380	958	Fig. 4 a	47	0392PM	<20	645	408
23	366NN-1	<20	381	816	Fig. 4 a	48	259KK	<20	274	644
24	274KO	<20	787	386	Fig. 4 a	49	1062KC	<20	113	523
25	276KT	<20	809	683	Fig. 4 a	50	031BR	<20	936	610
					51	472SD	<20	689	469	Fig. 7
					52	293KP-1	<20	1199	856	Fig. 7
					53	0557VP	<20	341	902	Fig. 7

Table S1: Parameters of HIV infected patients. Immunological and virological parameters of HIV patients, whose **Plasma** or **PBMC** were analyzed in this study.

Table S2.

Sample name	Sample Volume	Cell number	Purification method	Total protein concentration of EV
Plasma - Healthy	30 ml	-	Ultracentrifugation + Sucrose gradient	19.9 µg
Plasma - vir. HIV low CD4	30 ml	-	Ultracentrifugation + Sucrose gradient	194.8 µg
Plasma - non-vir. HIV high CD4	30 ml	-	Ultracentrifugation + Sucrose gradient	125.4 µg
Plasma - vir. HIV low CD4	30 ml	-	Ultracentrifugation + Sucrose gradient	160.2 µg
Jurkat T	60 ml	approx. 2.0×10^7	Ultracentrifugation	102.48 µg
Jurkat T + HIV	60 ml	approx. 2.0×10^7	Ultracentrifugation	140.55 µg
PBMC control (n=4)	70 ml	8.2×10^7	Ultracentrifugation	130.50 µg
PBMC HIV (n=7)	70 ml	3.0×10^7	Ultracentrifugation	120.30 µg

Table S2: Experimental details of Fig. 2, 3 and 7. pEV protein yield after sucrose gradient purification from plasma and cell culture supernatants used for CCF analysis.